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ANTISENSE HUMAN FUCOSYLTRANSFERASE SEQUENCES
AND METHODS OF USE THEREOF

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Related Applications

This application claims priority from U.S. Provisional Application Serial No.

10   60/131,068, filed April 26, 1999, the disclosure of which is incorporated by reference

herein in its entirety.

Statement of Federal Support

This invention was made with Government support under NIH-NCI grant

15   numbers 5K08-CA01758-05 and 1R55-CA755528-01. The Government has certain

rights to this invention.

Field of the Invention

The present invention concerns oligonucleotides, including antisense

20   oligonucleotides, that are useful in the treatment of cancer, along with DNA encoding

the same, vectors that contain such DNA, cells that express such DNA,

pharmaceutical formulations containing the same, and methods of use thereof in the

treatment of cancer.

25   ## Background of the Invention

Antisense oligonucleotides (along with other oligonucleotides that

downregulate gene expression) offer the possibility of targeting a specific gene to

study its function, inhibit its product, or repair a defect (A. Gewirtz et al., *Facilitating

oligonucleotide delivery: helping antisense deliver on its promise.* Proc. Natl. Acad.

30   Sci. USA, **93**: 3161-3163, 1996; L. Long et al., *Loss of the metastatic phenotype in

murine carcinoma cells expressing an antisense RNA to the insulin-like growth factor

receptor.* Cancer Res., **55**: 1006-1009, 1995; H. Sierakowska et al., *Repair of*

thalassemic human β-globin mRNA in mammalian cells by antisense oligonucleotides.
Proc. Natl. Acad. Sci. USA, **93**: 12840-12844, 1996). Antisense is therefore an
appealing therapeutic strategy for malignancies, as it avoids some of the widely
cytotoxic effects of current cancer therapy (J. Cheng et al., *Amplification of AKT2 in*
5   *human pancreatic cancer cells and inhibition of AKT2 expression and tumorigenicity*
by antisense RNA. Proc. Natl. Acad. Sci. USA, **93**: 3636-3641, 1996). Clinically,
antisense offers an advantage over antibody-based targeting (T. Kishimoto et al.,
Phenotypes correlating to metastatic properties of pancreas adenocarcinoma in vivo:
the importance of sialyl Lewis a antigen. Int. J. Cancer, **69**: 290-294, 1996) because
10   the constructs are often less immunogenic and have more favorable pharmacokinetics
(J. Phillips et al., *Pharmacokinetics, metabolism, and elimination of a 20-mer*
phosphorothioate oligodeoxynucleotide (CGP 69846A) after intravenous and
subcutaneous administration. Biochem. Pharmacol., **54**: 657-668, 1997).
Accordingly, there is a continued need for new oligonucleotides that have anticancer
15   activity.

Summary of the Invention

A first aspect of the present invention is an oligonucleotide that hybridizes to a
nucleic acid that encodes a fucosyltransferase (FUT). The fucosyltransferase is
20   preferably FUT3 or FUT6.

A second aspect of the present invention is a pharmaceutical formulation
comprising an oligonucleotide as described above in a pharmaceutically acceptable
carrier. The oligonucleotide is included in an amount effective to treat a cancer as
described herein, typically from about 0.01 percent to about 99 percent by weight of
25   the formulation.

A third aspect of the present invention is a method of treating a subject
afflicted with cancer (*e.g.*, a carcinoma), comprising administering to the subject an
oligonucleotide as described above in an amount effective to treat said cancer.

A fourth aspect of the present invention is a nucleic acid encoding an
30   oligonucleotide that hybridizes to a nucleic acid that encodes a fucosyltransferase.
The fucosyltransferase is preferably FUT3 or FUT6.

A fifth aspect of the present invention is a vector that contains and expresses a nucleic acid as described above.

A sixth aspect of the present invention is a pharmaceutical formulation comprising a vector as described above in a pharmaceutically acceptable carrier. The vector is included in an amount effective to treat cancer, typically from about 0.01 percent to about 99 percent by weight of the formulation.

A seventh aspect of the present invention is a method of treating a subject afflicted with cancer (e.g., a carcinoma), comprising administering to said subject a vector as described above in an amount effective to treat said cancer.

An eighth aspect of the present invention is a cell that contains and expresses a nucleic acid as described above.

The foregoing and other objects and aspects of the present invention are described in the figures herein and the specification set forth below.

Brief Description of the Drawings

Figure 1. Flow cytometry analysis of surface antigens on HT-29 and HT-29LMM cells. Cells were harvested, stained with monoclonal antibodies, and subjected to flow cytometric analysis as described in Materials and Methods. Anti-CD18 results are presented as negative controls. Data presented here are representative of the mean fluorescence intensity values from three separate experiments.

Figure 2. Fucosyltransferase specific activity of HT-29 and HT-29LMM cellular extracts. As detailed in Materials and Methods, Triton X-100 detergent extracts prepared from each cell line were assayed for their ability to transfer radiolabeled fucose to low molecular weight carbohydrate acceptors listed in the inset. Fucosyltransferase specific activity is expressed as mean pmol of fucose transferred per µg extract protein per hour, +/- standard errors, from four separate determinations.

Figure 3. Constructs used for stable transfection of HT-29LMM cells (Example 1) and COLO-205 cells (Example 2). The expression plasmid pcDNA3 contains a multi-cloning site downstream of the CMV promoter (CMVpr) and upstream of the neomycin resistance sequences (Neo). Constructs were assembled as described in Materials and Methods and linearized with PvuI before transfection.

Figure 8. Flow cytometry analysis of surface antigens on COLO-205 cells and stable transfectants. Cells were harvested, stained with monoclonal antibodies, and subjected to flow cytometric analysis as described in Materials and Methods. Anti-CD18 results are presented as negative controls. Data presented here are the
5 representative mean fluorescence intensity values from two experiments.

Figure 9. Fucosyltransferase specific activity of COLO-205 and transfectant cellular extracts. As detailed in Materials and Methods, Triton X-100 detergent extracts prepared from each cell line were assayed for their ability to transfer radiolabeled fucose to low molecular weight carbohydrate acceptors listed in the inset.
10 Control assays with no added acceptor were also performed (data not shown). Fucosyltransferase specific activity is expressed as pmol of fucose transferred per µg extract protein per hour.

Figure 10. Proliferative rate of COLO-205 and transfectant cell lines in RPMI with 10% FBS. For clarity, error bars are not shown for pcDNA3-CAT
15 transfectants. Antisense transfectants AS5C and AS7A (dotted lines) grew 3- to 7-fold slower than parental COLO-205 at day 7.

Figure 11 illustrates FUT3 and FUT6 sequence determinants used for the design of antisense oligonucleotides. Primers 3.1-3.4, 6.1-6.4 and 6.6-6.8 *See* H. Cameron et al., *J. Biol. Chem* **270**, 20112-20122 (1995); Primers 3.8 and 3.9, *see* B.
20 Weston et al., *J. Biol. Chem.* **267**, 24575 (1992); Primers 3.6 and 3.7 (underlined in the Figure) are identical in both genes. "CD" means catalytic domain.

Detailed Description of Preferred Embodiments

Amino acid sequences disclosed herein are presented in the amino to carboxy
25 direction, from left to right. The amino and carboxy groups are not presented in the sequence. Nucleotide sequences are presented herein by single strand only, in the 5' to 3' direction.

The production of cloned genes, recombinant DNA, recombinant vectors, proteins and protein fragments by genetic engineering is well known, and can be
30 carried out in accordance with known techniques. See, e.g., U.S. Patent No. 5,585,269 to Earp et al.; U.S. Patent No. 5,468,634 to Liu; and U.S. Patent No.

5,629,407 to Xiong et al. (the disclosures of all United States Patent references cited herein are to be incorporated herein by reference in their entirety) .

The fucosyltransferases FUT 3 and FUT6 referred to herein are known and can be obtained in accordance with known procedures. *See, e.g.,* B. Weston et al., *J. Biol. Chem.* **267**, 24575 (1992); R. McCurley et al., *Genomics* **26**, 142 (1995); H. Cameron et al., *J. Biol. Chem.* **270**, 20112 (1995). The gene may be of any species depending upon the subject and/or the particular use thereof, but is typically mammalian and is preferably human.

The term "target nucleic acid" as used herein refers to a nucleic acid (DNA or RNA) that encodes fucosyltransferase FUT3 or FUT6, as described above.

The term "treat" as used herein refers to any type of treatment that imparts a clinical improvement in the condition of the patient, or delays the progression of the disease.

The term "cancer" as used herein refers to any type of cancer, particularly solid tumors and preferably carcinomas. Specific cancers that may be treated by the method of the invention include colon, pancreatic, ovarian, gastric, breast, lung, heptaocellular, prostate, bladder, renal cell, and uterine cancer. Pancreatic, colon, ovarian and gastric cancer are preferred. And colon and pancreatic cancer are particularly preferred.

While the present invention is primarily concerned with the treatment of human subjects, the invention may also be carried out on animal subjects such as dogs, cats, and horses for veterinary purposes.

1. Oligonucleotides and administration.

The oligonucleotide that binds to the nucleic acid (*e.g.*, DNA, mRNA) may be any oligonucleotide that inhibits the expression of the target gene in cells infected by the vector, such as *(i)* an antisense oligonucleotide that specifically binds to the mRNA of the target gene to disrupt or inhibit translation thereof, *(ii)* a ribozyme that specifically binds to the target gene mRNA to disrupt or inhibit translation thereof, *(iii)* an oligonucleotide that specifically binds to the target nucleic acid duplex to form a triplex and disrupts or inhibits transcription or translation thereof, etc. All of these may be carried out in accordance with known techniques, as (for example) described

in U.S. Patents Nos. 5,650,316; 5,176,996, or 5,650,316 for triplex compounds, in

U.S. Patents Nos. 5,811,537; 5,801,154; and 5,734,039 for antisense compounds, and

in U.S. Patents Nos. 5,817,635; 5,811,300; 5,773,260; 5,766,942; 5,747,335; and

5,646,020 for ribozymes (the disclosures of which are incorporated by reference

5   herein in their entirety).

        The length of the oligonucleotide is not critical so long as the intended

function is achieved, but the oligonucleotide is typically from 5, 8, 10, 15, 18 or 20

nucleic acids in length up to 100, 200, 500 or 1000 nucleic acids in length, up to a

length equal the full length of the target gene.

10   The oligonucleotide may be natural (*e.g.,* RNA, DNA) or synthetic (*e.g.,* a

phosphorothioate compound). The oligonucleotide may be one that activates RNase

H (*e.g.,* and causes cleavage of the target molecules by RNase H) or one that does not

activate RNase H (*e.g.,* and operates by steric hinderance or blocking of the

transcription or translation mechanism). Chemical analogs of oligonucleotides with

15   modified or substituted phosphodiester residues, e.g., to the methylphosphonate, the

phosphotriester, the phosphorothioate, the phophorodithioate, or the

phosphororamidate, may be used. The oligonucleotides may be protected from

degredation by adding an end cap which is nuclease resistant, as described in J. Shaw

et al., *Nucleic Acids Res.* 19, 747 (1991). The oligonucleotide may be a morpholino

20   oligonucleotide, such as described in J. Summerton and D. Weller, *Antisense &*

Nucleic Acid Drug Dev. 7, 187-195 (1997). The design of specific binding sites on

the target gene or nucleic acid gene products (*e.g.,* mRNA) may be carried out in

accordance with known techniques. For example, the oligonucleotide may be

designed to have a sequence that binds to an intron/exon junction, or sufficiently close

25   to an intron/exon junction to inhibit the splicing-out of the intervening exon during

processing of precursor mRNA to mature mRNA).

        Oligonucleotides that do not activate RNase H can be made in accordance

with known techniques. See, e.g., U.S. Pat. No. 5,149,797 to Pederson et al. (The

disclosures of all patent references cited herein are to be incorporated herein by

30   reference in their entirety). Such oligonucleotides, which may be deoxyribonucleotide

or ribonucleotide sequences, simply contain any structural modification which

sterically hinders or prevents binding of RNase H to a duplex molecule containing the

oligonucleotide as one member thereof, which structural modification does not substantially hinder or disrupt duplex formation. Because the portions of the oligonucleotide involved in duplex formation are substantially different from those portions involved in RNase H binding thereto, numerous oligonucleotides which do

5   not activate RNase H are available. For example, such oligonucleotides may be oligonucleotides wherein at least one, or all, of the internucleotide bridging phosphate residues are modified phosphates, such as methyl phosphonates, methyl phosphonothioates, phosphoromorpholidates, phosphoropiperazidates and phosphoramidates. For example, every other one of the internucleotide bridging

10   phosphate residues may be modified as described. In another non-limiting example, such antisense oligonucleotides are oligonucleotides wherein at least one, or all, of the nucleotides contain a 2' loweralkyl moiety (e.g., C1-C4, linear or branched, saturated or unsaturated alkyl, such as methyl, ethyl, ethenyl, propyl, 1-propenyl, 2-propenyl, and isopropyl). For example, every other one of the nucleotides may be modified as

15   described. See also P. Furdon et al., *Nucleic Acids Res.* **17**, 9193-9204 (1989); S. Agrawal et al., *Proc. Natl. Acad. Sci. USA* **87**, 1401-1405 (1990); C. Baker et al., *Nucleic Acids Res.* **18**, 3537-3543 (1990); B. Sproat et al., *Nucleic Acids Res.* **17**, 3373-3386 (1989); R. Walder and J. Walder, *Proc. Natl. Acad. Sci. USA* **85**, 5011-5015 (1988).

20   The oligonucleotide may function to downregulate expression of the target nucleic acid by upregulating expression of an alternate, non-functional splice variant, in the manner described in U.S. Patent No. 5,665,593 to Kole.

         Figure 11 illustrates FUT3 and FUT6 sequence determinants used for the design of antisense oligonucleotides. Primers 3.1-3.4, 6.1-6.4 and 6.6-6.8 *See* H.

25   Cameron et al., *J. Biol. Chem* **270**, 20112-20122 (1995); Primers 3.8 and 3.9, *see* B. Weston et al., *J. Biol. Chem.* **267**, 24575 (1992); Primers 3.6 and 3.7 (underlined in the Figure) are identical in both genes. "CD" means catalytic domain. Thus, specific examples of antisense oligonucleotides to the FUT3 gene sequence include, but are not limited to, the following

30   3.1:   AGGCCATGGCAGGTTTCCTG (SEQ ID NO: 1)
          3.2:   AACTGAAGATCTACAAAAGA (SEQ ID NO: 2)
          3.3:   ACCAAGGTTCTGGAAAGAGA (SEQ ID NO: 2)

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3.4:   TGTAGGTCACCTGAGTGTGA  (SEQ ID NO: 4)

3.5:   GCTGCACCCAGGGGATCCAT  (SEQ ID NO: 5)

3.6:   TCTCGTAGTTGCTTCTGCTG  (SEQ ID NO: 6)

3.7:   GAGCGAGGCCGCAGCGTCTC  (SEQ ID NO: 7)

3.8:   ATCAGCCAGAACCATCACTC  (SEQ ID NO: 8)

3.9:   ACCTGTACCCTATAAGTGGT  (SEQ ID NO: 9)

3.A:   GATAACTTACCTGGAGAGGC  (SEQ ID NO: 10)

3.B:   TTAGGGTTGGACATGATATC  (SEQ ID NO: 11)
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Specific examples of antisense oligonucleotides to the FUT6 gene sequence include, but are not limited to, antisense oligonucleotides having the following sequences:

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6.1:   CCCACTCCTGCAGGGCAGTG  (SEQ ID NO: 12)

6.2:   GGGTCTTCACCACTGGAGAG  (SEQ ID NO: 13)

6.3:   AGTGAAAAGGCTGACCTGAA  (SEQ ID NO: 14)

6.4:   TGGATGCCCGTGACACTGGG  (SEQ ID NO: 15)

6.5:   GCCGGGCCCAGGGGATCCAT  (SEQ ID NO: 16)

6.6:   CACCCAGATCCAGCGTCCCA  (SEQ ID NO: 17)

6.7:   ATCTCCTGACCTTGTGATCC  (SEQ ID NO: 18)

6.8:   GATCTCCTGACCTAGGAAGA  (SEQ ID NO: 19)

6.A:   TTCTCACTCAGTTGGCCCAT  (SEQ ID NO: 20)

6.B:   CCAACCACCACACCTGTCAT  (SEQ ID NO: 21)

6.C:   GGACGAGTAACAGCTGGATT  (SEQ ID NO: 22)
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Additional examples of antisense oligonucleotides that may be used to carry out the present invention include, but are not limited to, the following:

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GCTTGGCTGCACCCAGGGGATC  (SEQ ID NO: 23) (FUT3 3.5); and

CTCTGCCGCTCCTGGACACTGCTGC  (SEQ ID NO: 24) (FUT 6
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LEADER).

Additional examples of antisense oligonucleotides that can be used to carry out the present invention include any continuous 15 or 18 nucleotide fragment of the sequences listed above.

The term "oligonucleotide" includes the physiologically and pharmaceutically acceptable salts thereof: i.e., salts that retain the desired biological activity of the parent compound and do not impart undesired toxicological effects thereto. Examples

of such salts are (a) salts formed with cations such as sodium, potassium, NH_4^+, magnesium, calcium, polyamines such as spermine and spermidine, etc.; (b) acid addition salts formed with inorganic acids, for example hydrochloric acid, hydrobromic acid, sulfuric acid, phosphoric acid, nitric acid and the like; (c) salts

5   formed with organic acids such as, for example, acetic acid, oxalic acid, tartaric acid, succinic acid, maleic acid, fumaric acid, gluconic acid, citric acid, malic acid, ascorbic acid, benzoic acid, tannic acid, palmitic acid, alginic acid, polyglutamic acid, naphthalenesulfonic acid, methanesulfonic acid, p-oluenesulfonic acid, naphthalenedisulfonic acid, polygalacturonic acid, and the like; and (d) salts

10   formed from elemental anions such as chlorine, bromine, and iodine.

Formulations of the present invention comprise the oligonucleotide in a physiologically or pharmaceutically acceptable carrier, such as an aqueous carrier. Thus, formulations for use in the present invention include, but are not limited to those suitable for parenteral administration, including subcutaneous, intradermal,

15   intramuscular, intravenous and intraarterial administration, as well as topical administration (*e.g.*, administration of an aerosolized formulation of respirable particles to the lungs of a patient). The formulations may conveniently be presented in unit dosage form and may be prepared by any of the methods well known in the art. The most suitable route of administration in any given case may depend upon the

20   subject, the nature and severity of the condition being treated, and the particular active compound that is being used.

The present invention provides for the use of oligonucleotides having the characteristics set forth above for the preparation of a medicament for a patient afflicted with cancer, as discussed above. In the manufacture of a medicament

25   according to the invention, the oligonucleotide is typically admixed with, among other things, an acceptable carrier. The carrier must, of course, be acceptable in the sense of being compatible with any other ingredients in the formulation and must not be deleterious to the patient. The carrier may be a solid or a liquid. One or more oligonucleotides may be incorporated in the formulations of the invention, which may

30   be prepared by any of the well known techniques of pharmacy consisting essentially of admixing the components, optionally including one or more accessory therapeutic ingredients.

Formulations of the present invention may comprise sterile aqueous and non-aqueous injection solutions of the active compound, which preparations are preferably isotonic with the blood of intended recipient and essentially pyrogen free. These preparations may contain anti-oxidants, buffers, bacteriostats and solutes which

5   render the formulation isotonic with the blood of the intended recipient. Aqueous and non-aqueous sterile suspensions may include suspending agents and thickening agents. The formulations may be presented in unit dose or multi-dose containers, for example sealed ampoules and vials, and may be stored in a freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid carrier, for example, saline

10   or water-for-injection immediately prior to use.

In the formulation the oligonucleotide may be contained within a lipid particle or vesicle, such as a liposome or microcrystal, which may be suitable for parenteral administration. The particles may be of any suitable structure, such as unilamellar or plurilamellar, so long as the antisense oligonucleotide is contained therein. Positively

15   charged lipids such as N-[1-(2,3-dioleoyloxi)propyl]-N, N,N-trimethyl-amoniummethylsulfate, or "DOTAP," are particularly preferred for such particles and vesicles. The preparation of such lipid particles is well known. See, e.g., U.S. Pat. Nos.4,880,635 to Janoff et al.; 4,906,477 to Kurono et al.; 4,911,928 to Wallach; 4,917,951 to Wallach; 4,920,016 to Allen et al.; 4,921,757 to Wheatley et al.; etc.

20   The dosage of the oligonucleotide administered will depend upon the particular method being carried out and when it is being administered to a subject, and will further depend on the disease, the condition of the subject, the particular formulation, the route of administration, etc. In general, intracellular concentrations of the oligonucleotide of from 0.05 or 0.2 µM, to about 5 or 50 µM, are desired. For

25   administration to a subject such as a human, a dosage of from about 0.01, 0.1, or 1 mg/Kg up to 50, 100, or 150 mg/Kg is employed.

2. Vectors and Vector administration.

Vectors used to carry out the present invention are, in general, RNA virus or

30   DNA virus vectors, such as lentivirus vectors, alphavirus vectors, papovavirus vectors (e.g., SV40 vectors and polyoma vectors), adenovirus vectors and adeno-associated virus vectors. See generally T. Friedmann, Science 244, 1275 16 (June 1989).

Examples of lentivirus vectors that may be used to carry out the present invention include Moloney Murine Leukemia Virus vectors, such as those described in U.S. Patent No. 5,707,865 to Kohn.

5 Any adenovirus vector can be used to carry out the present invention. *See, e.g.,* U.S. Patent No. 5,518,913, U.S. Patent No. 5,670,488, U.S. Patent No. 5,589,377; U.S. Patent No. 5,616,326; U.S. Patent No. 5,436,146; and U.S. Patent No. 5,585,362. The adenovirus can be modified to alter or broaden the natural tropism thereof, as described in S. Woo, *Nature Biotechnology* 14, 1538 (Nov. 1996).

Any adeno-associated virus vector (or AAV vector) can also be used to carry 10 out the present invention. *See, e.g.,* U.S. Patent No. 5,681,731; U.S. Patent No. 5,677,158; U.S. Patent No. 5,658,776; U.S. Patent No. 5,658,776; U.S. Patent No. 5,622,856; U.S. Patent No. 5,604,090; U.S. Patent No. 5,589,377; U.S. Patent No. 5,587,308; U.S. Patent No. 5,474,935; U.S. Patent No. 5,436,146; U.S. Patent No. 5,354,678; U.S. Patent No. 5,252,479; U.S. Patent No. 5,173,414; U.S. Patent No. 15 5,139,941; and U.S. Patent No. 4,797,368.

Alphavirus vectors are known and described in, for example, U.S. Patent No. 5,811,407 to Johnston et al. and 5,505,947 to Johnston et al.

The regulatory sequences, or the transcriptional and translational control sequences, in the vectors can be of any suitable source, so long as they effect 20 expression of the heterologous nucleic acid in the target cells. For example, commonly used promoters are the LacZ promoter, and promoters derived from polyoma, Adenovirus 2, and Simian virus 40 (SV40). See, e.g., U.S. Patent No. 4,599,308.

The heterologous nucleic acid may encode any product that inhibits the 25 expression of the target gene in cells infected by the vector, such as an antisense oligonucleotide that specifically binds to the mRNA of the target gene to disrupt or inhibit translation thereof, a ribozyme that specifically binds to the target gene mRNA to disrupt or inhibit translation thereof, or a triplex nucleic acid that specifically binds to the target gene duplex DNA and disrupts or inhibits transcription thereof, as 30 described above. Again, the length of the heterologous nucleic acid is not critical so long as the intended function is achieved, but the heterologous nucleic acid is

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typically from 5, 8, 10 or 20 nucleic acids in length up to 100, 200, 500 or 1000 nucleic acids in length, up to a length equal the full length of the target gene.

Once prepared, the recombinant vector can be reproduced by *(a)* propagating the vector in a cell culture, the cell culture comprising cells that permit the growth and
5   reproduction of the vector therein; and then *(b)* collecting the recombinant vector from the cell culture, all in accordance with known techniques. The viral vectors collected from the culture may be separated from the culture medium in accordance with known techniques, and combined with a suitable pharmaceutical carrier for administration to a subject. Such pharmaceutical carriers include, but are not limited
10   to, sterile pyrogen-free water or sterile pyrogen-free saline solution. If desired, the vectors may be packaged in liposomes for administration, in accordance with known techniques.

Any suitable route of administration can be used to carry out the present invention, depending upon the particular condition being treated. Suitable routes
15   include, but are not limited to, intraveneous, intrarterial, intrathecal, intraperitoneal, intramuscular, and intralesional injection. Intralesional injection is currently preferred.

The dosage of the recombinant vector administered will depend upon factors such as the particular disorder, the particular vector chosen, the formulation of the
20   vector, the condition of the patient, the route of administration, etc., and can be optimized for specific situations. In general, the dosage is from about 10^7, 10^8, or 10^9 to about 10^{11}, 10^{12}, or 10^{13} plaque forming units (pfu).

In addition to the foregoing, the present invention provides cells, particularly mammalian and most preferably human, cells that contain and express an
25   oligonucleotide as described above. The cells are useful, among other things, for screening oligonucleotides for their ability to bind to the target nucleic acids described herein. Such cells may be transiently or stably transfected; that is, may transiently or stably express the oligonucleotides described herein. Vectors as described above may be employed, or other vectors such as plasmid vectors, in accordance with known
30   techniques.

The present invention is described in further detail in the following non-limiting Examples, in which the following abbreviations are used: **Lewis x**, Galβ1-

>4[Fucα1->3]GlcNAc; **sLex, sialyl Lewis x,** NeuNAcα2->3Galβ1->4[Fucα1->3]GlcNAc; **sLea, sialyl Lewis a,** NeuNAcα2->3Galβ1->3[Fucα1->4]GlcNAc; **LacNAc,** N-acetyllactosamine, Galβ1->4GlcNAc; **Lacto-N-biose I,** Galβ1->3GlcNAc; **sialyl LacNAc,** α(2,3)sialyl-N-acetyllactosamine, NeuNAcα2->3Galβ1-

5   >4GlcNAc; **α(1,3)fucosyltransferase,** GDP-fucose:β-D-N-acetylglucosaminide 3-α-L-fucosyltransferase; **PCR,** polymerase chain reaction; **RT-PCR,** reverse transcriptase-mediated polymerase chain reaction; **kb,** kilobase; **bp,** base pair; **FBS,** fetal bovine serum; **TBS,** tris-buffered saline; **PBS,** phosphate-buffered saline; **FITC,** fluorescein isothiocyanate; **HUVEC,** human umbilical vein endothelial cell; **TNF-α,**

10   tumor necrosis factor-α.

EXAMPLE 1

Expression of human α(1,3)fucosyltransferase antisense
sequences inhibits selectin-mediated adhesion

15   ### and liver metastasis of colon carcinoma cells

The initial steps of leukocyte and tumor cell adhesion involve selectin receptor/ligand interactions. The selectin ligand components sialyl Lewis x and sialyl Lewis a are oncodevelopmental antigens involved in progression of adenocarcinoma. Interrupting biosynthesis of these surface glycans by inhibition of

20   α(1,3)fucosyltransferase (FUT) gene expression is an attractive goal for functional and therapeutic studies. We report here the inhibition of E-selectin-mediated adenocarcinoma cell adhesion by stable transfection of antisense sequences directed at the human Lewis α(1,3/1,4) fucosyltransferase gene, FUT3. The metastatic parental cell line, HT-29LMM, expressed high levels of sialyl Lewis x, sialyl Lewis a,

25   α(1,3/1,4)fucosyltransferase activity, and FUT3 transcript, but antisense transfectant cell lines did not. When injected into the spleens of nude mice, the stable antisense clones were unable to colonize the liver. These results provide target validation for inhibition of carcinoma metastasis with antisense FUT sequences and confirm the primacy of α(1,3)fucosyltransferases in the synthesis of selectin ligands.

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A. MATERIALS AND METHODS

Cell culture. HT-29, HT-29LMM, and human umbilical endothelial cells (HUVEC) were isolated and propagated as previously described (J. Price et al., *Clin. Expl. Metastasis*, 7: 55-68, 1989; K. Yoshida et al., *J. Surg. Oncol.*, 49: 249-252, 1992; L. Romer et al., *Mol. Biol. Cell.*, 5: 349-361, 1994).

Antibodies. Mouse IgG1/IgG2 control and anti-CD18 antibodies were obtained through Becton Dickinson (San Jose, CA). Anti-Lewis x antibody (anti-SSEA-1, mouse IgM) was obtained through the Developmental Studies Hybridoma Bank (Iowa City, IA). A monoclonal anti-VIM-2 antibody (IgM) was purchased from Immunotech (Westbrook, ME). Monoclonal anti-sialyl Lewis a antibody (CSLEA-1, mouse IgG3) was a gift from Dr. P. Terasaki (UCLA, Los Angeles) Sources for the anti-sialyl Lewis x IgM monoclonal antibodies CSLEX1 and KM93 were Becton Dickinson and Kamiya Biomedical (Seattle, WA), respectively. For blocking studies, anti-E-selectin antibody (CD62E, 1.2B, mouse IgG1) was obtained from Serotec (Raleigh, NC). Fluorescein-conjugated goat anti-mouse IgM and IgG antibodies were purchased from Sigma (St. Louis, MO).

Flow cytometry analysis. HT-29, HT-29LMM, and cloned cell lines were prepared and stained with monoclonal anti-carbohydrate antibodies at saturating concentrations as described (B. Weston et al., *J. Biol. Chem.*, 267: 4152-4160, 1992; B. Weston et al., *J. Biol. Chem.*, 267: 24575-24584, 1992). Anti-SSEA-1 was used at a dilution of 1:1000 and anti-VIM-2 was used at 1:50. Anti-sialyl Lewis x (CSLEX-1) was used at 10 μg/ml. Anti-sialyl Lewis a (CSLEA-1) was used at 1:500. Cells were then stained with fluorescein isothiocyanate-conjugated goat anti-mouse IgM or anti-mouse IgG. Mouse IgG1/IgG2 and anti-CD18 antibodies (negative controls throughout) were used according to the manufacturer's instructions. Cells were analyzed on a Becton-Dickinson FACScan as previously described (*Id.*).

Fucosyltransferase assays. Triton X-100 extracts were prepared from HT-29, HT-29LMM, and transfected cells as described (*Id.*). Fucosyltransferase assays were performed in a volume of 20 μl, and contained 25 mM sodium cacodylate (pH 6.2), 5 mM ATP, 10 mM L-fucose, 20 mM MnCl$_2$, 3 μM GDP-[^{14}C]fucose (Amersham), and 10 μg of cell extract protein. Acceptor substrates

(N-acetyllactosamine, Galβ1->4GlcNAc; Lacto-N-biose I, Galβ1->3GlcNAc; or α(2,3)sialyl-N-acetyllactosamine, NeuNAcα2->3Galβ1->4GlcNAc) were added to a final concentration of 20 mM. Control assays with no added acceptor were performed using the same conditions. Reactions were incubated at 37° C for one hour. Assays were terminated with 580 μl of 5 mM sodium phosphate, pH 6.8. The terminated assays were centrifuged and the supernatants were collected. An aliquot of each terminated reaction supernatant was subjected to scintillation counting. Another aliquot was applied to a column containing either Dowex 1X2-400, formate form or a Dowex 1-X8 (PO4^{-2}) column equilibrated as previously described (*Id.*). To quantitate incorporation of radioactive fucose into product, the flow-through fraction and 2 ml of a subsequent water elution were collected, pooled, and counted. A portion of this material was assayed by HPLC as described (*Id.*).

Sialyltransferase assays. Sialyltransferase assays were performed in a volume of 30 μl, and contained 50 mM Tris-maleate (pH 6.7), 10 mM MnCl$_2$, 0.3 nM CMP-[^{14}C]NeuNAc (Amersham), and 10 μg of cell extract protein. Acceptor substrate (N-acetyllactosamine, Galβ1->4GlcNAc, Sigma) was added to a final concentration of 0.1 mM. Control assays with no added acceptor were performed using the same conditions. Reactions were incubated at 37° C for two hours. Assays were terminated by addition of 30 μl ethanol, followed by addition of 540 μl of distilled water. The terminated assays were desalted over Dowex AG 1 (AC-) and Dowex AG 50 (H+). Neutral saccharides were eluted with 4 ml of water and charged saccharides with 14 ml of 0.5 M acetic acid (M. Majuri et al., Int. J. Cancer, *63:* 551-559, 1995). An aliquot of each reaction was subjected to scintillation counting. Another aliquot was used for HPLC confirmation of product structure in accordance with known techniques.

RNA isolation. Total cellular RNA was extracted with guanidine isothiocyanate and purified by cesium chloride gradient centrifugation. Poly(A)$^+$ RNA was prepared using oligo(dT)-cellulose chromatography (H. Cameron et al., J. Biol. Chem., *270:* 20112-20122, 1995).

Northern blot analysis. Poly(A)$^+$ RNA (15 ug, divided into 5 μg per lane) was denatured and separated with 1.2% formaldehyde agarose gel electrophoresis and

transferred to Hybond-N membranes (Amersham). The membrane was cut into separate strips, which were then prehybridized for 4 hours at 42 °C. Gene-specific probes were constructed as previously described, and hybridization to β-actin message was used as control (S. Natsuka et al., *J. Biol. Chem.,* **269**: 16789-16794,

5   1994; H. Cameron et al., *J. Biol. Chem.,* **270**: 20112-20122, 1995; K. Yago et al., *Cancer Res.,* **53**: 5559-5565, 1993.). Probes were labeled with [^{32}P]dCTP by random priming and purified from unincorporated isotope at a specific activity of 1 x 10^9 cpm/ug or higher (M. Majuri et al., *supra.).* Hybridization was carried out at 42 °C for 16-20 h. The final wash was carried out at 65 °C with 0.5 X SSC and 0.2% SDS

10   for 30 min. Autoradiograms were scanned and the images were sized, grouped, and labeled using Adobe Photoshop 5.0 (Mountain View, CA).

RT-PCR analysis. First strand cDNA was prepared using 200 ng of poly(A)$^+$ RNA. Synthesis of cDNA was carried out in a 50 μL reaction volume with 12 U of Moloney murine leukemia virus reverse transcriptase and 125 ng of lower strand

15   cDNA primers (H. Cameron et al., *supra*). PCR amplifications were performed using the lower strand cDNA primers and previously described FUT3-FUT7 gene-specific upper strand primers and amplification profiles (K. Sasaki et al., J. Biol. Chem., *269:* 14730-14737, 1994.; H. Cameron et al., *supra*). To control for genomic DNA contamination, parallel amplifications of all samples with no reverse transcriptase were

20   performed (data not shown). RT-PCR of human cytoplasmic β-actin was performed with previously reported primers to verify the quality/quantity of RNA and further exclude the possibility of genomic contamination in accordance with known techniques.

Construction of FUT3 antisense, sense, and control plasmids for stable

25   **transfection of HT-29LMM.** The plasmid pcDNA3 (InVitrogen, Carlsbad, CA) was chosen for cloning and selection of FUT3 antisense, sense, and control constructs in HT-29LMM due to preliminary data showing high level expression of chloramphenicol acetyltransferase (CAT) in stable transfection experiments of parental HT-29 cells (data not shown). The CAT coding region (Pharmacia,

30   Piscataway, NJ) was cloned in the sense orientation into the *Hind*III site of pcDNA-3 and served as control throughout expression studies. The plasmid pcDNA3-FUT3S

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was created by digestion of pFUT3 (R. Mollicone et al., J. Biol. Chem., *269:* 20987-20994, 1994) with *Xho*I and *Xba*I and directional cloning into pcDNA3. Likewise, pFUT3 was also digested with *Xho*I and *Hind*III, and the resulting fragment cloned in antisense orientation to the CMV promoter in pcDNA3, yielding the expression vector

5 pcDNA3-FUT3AS. Finally, a truncated coding region antisense construct was created by amplification of FUT3 bp 733-1004 (J. Kukowska-Latallo et al., Genes Devel., *4:* 1288-1303, 1990) with the *Hind*III-containing primers *aagctt*CTGGCCTTCGAGAACTCCTTGCACC (upper strand, *Hind*III in lower case) and *aagctt*AGTGCCCAGCTGAAGGAGCGAGGCC (lower strand). The resulting

10 272 bp fragment, which corresponds to the putative catalytic domain of FUT3 (*Id.*), was cloned in reverse orientation into the *Hind*III site of pcDNA3 to yield the vector pcDNA3-FUT3CD AS (data not shown).

Stable transfection, selection, and cloning of FUT3 antisense and sense HT-29LMM cell lines. Approximately 24 hours before transfection, HT-29LMM

15 cells were plated at a density of 1.30 - 1.50 X 10^6 cells per 60 mm dish in 5 ml of RPMI-1640 with 10% FBS. In separate tubes, 8 μg of the *Pvu*I-linearized plasmids pcDNA3-CAT, pcDNA3-FUT3S, pcDNA3-FUT3AS, or pcDNA3-FUT3CD AS (above) were mixed with 25 μl of Lipofectamine reagent (GIBCO-BRL, Gaithersburg, MD) in 200 μl of serum-free media and incubated for 45 minutes at

20 room temperature to allow complex formation. The plates were washed twice with serum-free media, the DNA-liposome complexes were diluted with 800 μl of additional serum-free media, and the dilute complexes were added to each plate. The plates were incubated for 24 hours at 37^0C in 5% CO_2. The following day, the serum-free media and unincorporated liposomal complexes were replaced with 5 ml

25 of RPMI-1640 with 10% FBS. Forty-eight hours after transfection, G-418 sulfate (GIBCO-BRL) was added to the medium at a concentration of 1 mg/ml.

After 3-4 weeks in G-418, distinct colonies were observed on each plate and selected with 3 mm cloning cylinders (Fisher, Pittsburgh, PA). The colonies were allowed to grow, removed with trypsin, and cloned by limiting dilution in 96 well

30 plates with continued G-418 selection. To assure a homogeneous population, the resulting clones were again subjected to limiting dilution under G-418 selection.

Following expansion, cells from each well were subjected to the same expression studies described above. Five clonal sense, five clonal antisense, and three clonal CAT control cell lines were established. Representative clones of each were further characterized for glycan, enzyme, and transcript expression. The two sense clones

5   chosen for study were designated as LMM/S1 and LMM/S2. The two antisense clones selected were LMM/AS1 (transfected with pcDNA3-FUT3AS) and LMM/AS2 (transfected with pcDNA3-FUT3CD AS).

HUVEC adhesion assays. HUVEC, at passage 2, were plated in 30 mm plates and allowed to grow to confluence two days prior to use in cell adhesion

10   assays. Half of the HUVEC plates were incubated with TNFα (20 ng/ml) in medium 199 with 10% fetal bovine serum for 7 hours, while the remaining half were not treated with cytokine. HT-29, HT-29LMM, and transfected cells were washed, fixed, and added to HUVEC monolayers as described (J. Lowe et al., Cell, 63: 475-484, 1990). After settling, the cells were incubated on the monolayers for 45 minutes at

15   6°C. The cells were then removed by gentle aspiration followed by three media washes as described (*Id.*). The number of cells recovered from each plate was determined by counting the pooled washes; the number of bound cells represents the difference between the number of cells applied to each plate and the number recovered from each plate. For blocking with anti-E-selectin antibody, HUVEC were

20   preincubated with 20 μg/ml antibody for 30 minutes at 37°C.

Establishment of hepatic metastases. NCR nu/nu athymic nude mice were obtained from Taconic (Germantown, N.Y.). They were maintained in facilities approved by the American Association for Accreditation of Laboratory Animal Care under specific pathogen-free conditions and fed sterile food and water. Seven- to

25   eight-week-old female nude mice weighing 20-22 gm were anesthetized with intraperitoneal injections of NEMBUTAL™ pentobarbitol. Animals were placed in the supine position and prepared using aseptic technique. Under sterile conditions, a flank incision was made. The spleen was visualized and injected with 1×10^7 tumor cells in 100 μl of PBS. The contents were returned to the abdominal cavity and

30   surgical clips were used to close the incision. The mice were allowed to recover and surgical clips were removed from the animals upon complete healing of the

laparotomy incision. Liver metastases were grossly evident by 5-7 weeks. Animals were then sacrificed, and liver harvested, examined, and weighed. Livers were harvested from euthanized animals and placed in 10% formalin, and subsequently embedded in paraffin.

5

B. RESULTS

HT-29LMM cells express higher levels of sialyl Lewis x, sialyl Lewis a, and α(1,3)fucosyltransferase activity than parental HT-29 cells. Results of flow cytometry analyses of surface antigens on HT-29 and HT-29LMM cells are

10 summarized in **Fig. 1**. HT-29 and HT-29LMM cells do not express high levels of the non-sialylated antigen, Lewis x, or the internally fucosylated antigen, VIM-2 (data not shown). Higher surface levels of the fucosylated and sialylated selectin ligands sialyl Lewis a and sialyl Lewis x are seen on the derived cell line HT-29LMM. Previous reports have documented high expression of dimeric sialyl Lewis x in HT-29LMM (S.

15 Hoff et al., Arch. Surg., *125:* 206-209, 1990). To assess if this high level of surface glycan expression was due to corresponding increases in α(1,3/1,4)fucosyltransferase activity, enzyme assays with low molecular weight carbohydrate acceptors were performed. As shown in **Fig. 2**, cellular extracts from HT-29LMM transferred fucose more efficiently to both type I and type II acceptors (J. Kukowska-Latallo et al.,

20 Genes Devel., *4:* 1288-1303, 1990) when compared to parental cells. No difference was seen in sialyltransferase activity with HT-29LMM (data not shown). This antigenic and enzymatic profile is consistent with increased expression of Fuc-TIII (J. Kukowska-Latallo et al., *Cell* **63**, 1288 (1990); B. Weston, et al., *J. Biol. Chem.* **267**, 4152 (1992)) in HT-29LMM.

25 **HT-29LMM cells express higher levels of FUT3 transcript than parental HT-29 cells.** To assess whether surface expression of fucosylated ligands is specifically associated with increased expression of FUT3 in HT-29LMM, fucosyltransferase transcript analyses were performed. The results of Northern blot analyses of HT-29 and HT-29LMM cells showed higher levels of FUT3 transcript in

30 the derived cell line (data not shown). RT-PCR analysis of HT-29 and HT-29LMM cells confirms this increase in FUT3 expression (data not shown). Using similar amplification methods (K. Sasaki et al., J. Biol. Chem., *269:* 14730-14737, 1994; H.

Cameron et al., J. Biol. Chem., *270:* 20112-20122, 1995; K. Yago et al., Cancer Res., *53:* 5559-5565, 1993), expression of other human fucosyltransferase transcripts is low (FUT4, FUT6, FUT7) or not detectable (FUT5) in both cell lines (data not shown). FUT4-FUT7 transcripts were not detectable by hybridization (data not shown). FUT3 was therefore chosen as the primary target for antisense inhibition studies.

Characterization of HT-29LMM antisense, sense, and control cell lines. Constructs used for stable transfection of HT-29LMM cells are shown in **Fig. 3**. The plasmid pcDNA3-CAT was used throughout the following experiments to monitor expression levels over time by CAT assay (data not shown). The plasmid pcDNA3-FUT3S was transfected to allow quantitative comparison of fucosyltransferase activity in cell lines (**Table 1**) and to serve as an additional control for high level expression over time.

Enzyme assay results in **Table 1** show that transfection of the control plasmid pcDNA3-CAT did not affect fucosyltransferase activities. Stable expression of pcDNA3-FUT3S sense constructs in clones LMM/S1 and LMM/S2 enhanced fucosyltransferase activity. In contrast, the cloned antisense cell lines LMM/AS1 and LMM/AS2 had less than 2% specific activity with sialyl LacNac, the trisaccharide precursor for sialyl Lewis x, when compared to untransfected HT-29LMM cells. Furthermore, extracts from LMM/AS1 and LMM/AS2 showed marked reduction in the ability to fucosylate the Type 1 acceptor Lacto-*N*-biose I. No difference was seen in sialyltransferase activity for any of the cell lines or controls (data not shown). This profile is most consistent with reduced activity of Fuc-TIII in the antisense lines (J. Kukowska-Latallo et al., Genes Devel., *4:* 1288-1303, 1990; B. Weston et al., J. Biol. Chem., *267:* 4152-4160, 1992). Flow cytometry analyses of surface antigens on HT-29LMM and stable antisense transfectants (**Fig. 4**) confirm that the synthesis of selectin ligands sialyl Lewis x and sialyl Lewis a is inhibited in LMM/AS1 and LMM/AS2 cells. Northern blot and RT-PCR analyses show that FUT3 transcript levels are markedly reduced in both stable antisense transfectant lines (**Table 2**).

Stable transfection of HT-29LMM with FUT3 antisense constructs inhibits selectin-mediated cell adhesion. To assess whether inhibition of sialyl Lewis x and sialyl a expression on LMM/AS1 and LMM/AS2 was of functional significance, HT29, HT-29LMM and antisense transfectants were assayed for

adhesion to human umbilical vein endothelial cells (HUVEC, **Fig. 5**). Each cell line was tested for adhesion to TNF-α-stimulated HUVEC (striped bars) or untreated HUVEC (black bars) as detailed in Materials and Methods. Blocking with anti-E-selectin monoclonal antibody was performed as described (J. Lowe et al., Cell, *63*:

5   475-484, 1990; M. Phillips et al., Science, *250*: 1130-1132, 1990.). The number of bound cells was calculated by subtracting the number of cells recovered (per plate, in pooled washes) from the number of cells applied to each plate. As summarized in **Fig. 5**, HT-29LMM cells bound avidly to cytokine-stimulated HUVEC; this binding was specifically inhibited by pre-treatment of HUVEC with anti-E-selectin antibody.

10   The control cell line HT-29LMM/pcDNA3-CAT bound HUVEC with similar affinity (data not shown). Neither antisense cell line was capable of adhering to TNF-α-stimulated HUVEC *in vitro*. These cell lines were chosen for *in vivo* metastasis studies below.

Stable antisense transfectants of HT-29LMM do not metastasize to liver.

15   Splenic injections of nude mice with HT-29LMM and the antisense transfectant cell lines LMM/AS1 and LMM/AS2 were performed as described in Materials and Methods. Splenic tumors and production of experimental liver metastases were evaluated after 7 weeks. All injected mice developed tumors in the spleen. As shown in **Table 3**, all 8 mice injected with HT-29LMM developed liver metastases,

20   consistent with previously reported results (S. Hoff et al., Arch. Surg., *125*: 206-209, 1990; J. Price et al., Clin. Expl. Metastasis, *7*: 55-68, 1989; K. Yoshida et al., J. Surg. Oncol., *49*: 249-252, 1992). Average liver replacement by hepatic metastases of HT-29LMM was 48% (range 23-89%) and liver mass increased by approximately 2-fold (**Table 3**). None of the mice injected with LMM/AS1 or LMM/AS2 transfectants

25   developed hepatic metastasis by gross (**Fig. 6**) or microscopic (**Fig. 7**) examination.

TABLE 1. $\alpha(1,3)$-Fucosyltransferase activity in cellular extracts

Cells/Constructs	Acceptor	Activity (+/-SD) (*pmol/mg/hr*)
HT-29LMM	LacNac	4070 +/- 292*
	Sialyl-LacNac	2980 +/- 311
	Lacto-*N*-biose I	6230 +/- 324
HT-29LMM/ pcDNA3-CAT[t]	LacNac	4150 +/- 388
	Sialyl-LacNac	3300 +/- 275
	Lacto-*N*-biose I	5920 +/- 453
LMM/AS1	LacNac	152 +/- 20
	Sialyl-LacNac	34 +/- 13
	Lacto-*N*-biose I	85 +/- 41
LMM/AS2	LacNac	141 +/- 17
	Sialyl-LacNac	46 +/- 32
	Lacto-*N*-biose I	73 +/- 44
LMM/S1	LacNac	5510 +/- 477
	Sialyl-LacNac	4690 +/- 450
	Lacto-*N*-biose I	7160 +/- 606
LMM/S2	LacNac	5025 +/- 221
	Sialyl-LacNac	4270 +/- 340
	Lacto-*N*-biose I	6900 +/- 598

*Untransfected HT-29LMM results are from the same assays reported in Figure 2 (included here for comparison).

[t]CAT assays performed at same time points show stable expression of plasmid pcDNA3-CAT (data not shown).

HT-29LMM cells were transfected with constructs and methods described in "Experimental Procedures." Fucosyltransferase assays were performed on extracts from transfected cells 28, 56, and 84 days after G-418 selection, cloning, and expansion.

TABLE 2. Quantitation of FUT3 transcript levels in HT-29LMM
and antisense clones by Northern blot and RT-PCR.

Cell line	Expression level relative to β-actin
HT-29	0.2*
HT-29LMM	1.0*
LMM/AS1	<.05
LMM/AS2	<.05

*Comparison data not shown.
PolyA+ RNA from HT-29LMM cells and stable anti-sense transfectants was analyzed as described in "Experimental Procedures." Signal densities were compared using Adobe Photoshop 5.0.

TABLE 3. Liver metastases in nude mice following splenic injection
of HT-29LMM cells and stable anti-sense transfectant lines.

Cells/Constructs	Liver metastases	Liver weights (*mean;range*)
HT-29LMM	8/8	2.45 gm (2.00-3.25)*
LMM/AS1	0/8	1.26 gm (1.14-1.38)
LMM/AS2	0/8	1.33 gm (1.15-1.44)

*$p < .01$, HT-29LMM versus LMM/AS1, HT-29LMM versus LMM/AS2.
HT-29LMM cells were transfected with constructs, selected, and characterized for fucosylated glycan expression as described above. 10^7 tumor cells in 100 μl of PBS were injected into the spleen of an anesthetized nude mouse. The animals were sacrificed 7 weeks post-operatively or when liver metastases were easily palpable.

EXAMPLE 2

Transfection of α(1,3)fucosyltransferase antisense

sequences impairs the proliferative and tumorigenic

ability of human colon carcinoma cells

Sialyl Lewis x and sialyl Lewis a are oncodevelopmental antigens involved in the pathogenesis of colon adenocarcinoma. Interrupting biosynthesis of these glycans by disruption of α(1,3)fucosyltransferase gene expression is a potential approach to

molecular therapy. This example reports the disruption of sialyl Lewis x/a biosynthesis and inhibition of colon carcinoma cell proliferation by stable transfection of antisense sequences directed at the human Lewis $\alpha(1,3/1,4)$fucosyltransferase gene, FUT3, and the plasma $\alpha(1,3)$fucosyltransferase gene, FUT6. COLO-205 cells

5   expressed high levels of sialyl Lewis x/a, $\alpha(1,3)$fucosyltransferase activity, and FUT3/6 transcripts, but COLO-205-derived antisense transfectant cell lines AS5C and AS7A did not. Sense transfectants S6G expressed higher levels of FUT enzyme and transcript than parental COLO-205. Cellular proliferation assays showed marked correlative decreases in the growth of antisense lines and, conversely, increased

10   growth of sense transfectants. Subcutaneous tumors created by injection of nude mice with antisense transfectant cell lines grew more slowly than those arising from control COLO-205 and sense transfectants. These results provide target validation for inhibition of colon carcinoma proliferation with antisense sequences directed at human FUT genes.

15

A. MATERIALS AND METHODS

Cell culture. COLO-205 cells were propagated in RPMI-1640 with 10% FBS.

Antibodies. Antibodies were produced as described in Example 1 above.

20   **Flow cytometry analysis.** These procedures were carried out in essentially the same manner as described in Example 1 above, with COLO-205 and cloned cell lines were prepared and stained in like manner as described therein.

Fucosyltransferase assays. Triton X-100 extracts were prepared from parental COLO-205 and transfected cells as described in Example 1 above.

25   Fucosyltransferase assays were performed in a volume of 20 µl, and contained 25 mM sodium cacodylate (pH 6.2), 5 mM ATP, 10 mM L-fucose, 20 mM MnCl$_2$, 3 µM GDP-[^{14}C]fucose (Amersham), and 10 µg of cell extract protein. Acceptor substrates (*N*-acetyllactosamine, Galβ1->4Glc*N*Ac, Lacto-*N*-biose I, Galβ1->3Glc*N*Ac, or $\alpha(2,3)$sialyl-*N*-acetyllactosamine (Neu*N*Acα2->3Galβ1->4Glc*N*Ac,) were added to a

30   final concentration of 20 mM. Control assays with no added acceptor were performed using the same conditions. Reactions were incubated at 37° C for one hour. Assays

were terminated with 580 μl of 5 mM sodium phosphate, pH 6.8. The terminated

assays were centrifuged and the supernatants were collected. An aliquot of each

terminated reaction supernatant was subjected to scintillation counting. Another

aliquot was applied to a column containing either Dowex™ 1X2-400 (formate form)

5   or a Dowex™ 1-X8 (PO_4^{-2}) column equilibrated as described above. To quantitate

incorporation of radioactive fucose into product, the flow-through fraction and

subsequent water elution were collected, pooled, and counted. A portion of this

material was assayed by HPLC as described above.

        RNA isolation and Northern blot analyses. Total cellular RNA was

10   extracted with guanidine isothiocyanate and purified by cesium chloride gradient

centrifugation. Poly(A)$^+$ RNA was prepared using oligo(dT)-cellulose

chromatography, denatured, and separated with 1.2% formaldehyde agarose gel

electrophoresis (5 μg per lane) and transferred to Hybond-N™ membranes

(Amersham). The membrane was cut into separate strips, which were then

15   prehybridized for 4 hours at 42 °C. Gene-specific probes were amplified using

previously described PCR conditions and primer sets (H. Cameron et al., J. Biol.

Chem., *270:* 20112-20122, 1995). Hybridization to β-actin message was used as

control (*Id.*; K. Yago et al., Cancer Res., *53:* 5559-5565, 1993). Probes were labeled

with [^{32}P]dCTP by random priming and purified from unincorporated isotope at a

20   specific activity of 1 x 10^9 cpm/ug or higher. Hybridization was carried out at 42 °C

for 16-20 h. The final wash was carried out at 65 °C with 0.5 X SSC and 0.2% SDS

for 30 min. Autoradiograms were scanned and the images were sized, grouped, and

labeled using Adobe Photoshop 5.0 (Mountain View, CA).

        Construction of antisense, sense, and control plasmids for stable

25   **transfection of COLO-205.** The plasmid pcDNA3 (InVitrogen, Carlsbad, CA) was

chosen for cloning and selection of antisense, sense, and control constructs in COLO-

205 due to data showing high level expression of chloramphenicol acetyltransferase

(CAT) in previous transfections (data not shown). The CAT coding region

(Pharmacia, Piscataway, NJ) was cloned in the sense orientation into the *Hind*III site

30   of pcDNA-3 and served as control throughout expression studies. The plasmid

pcDNA3-FUT3S was created by digestion of pFUT3 (R. Mollicone et al., J. Biol.

Chem., *269:* 20987-20994, 1994) with *Xho*I and *Xba*I and directional cloning into pcDNA3. Likewise, pFUT3 was also digested with *Xho*I and *Hind*III, and the resulting fragment cloned in antisense orientation to the CMV promoter in pcDNA3, yielding the expression vector pcDNA3-FUT3AS. The FUT6 sense plasmid

5   pcDNA3-FUT6S and antisense plasmid pcDNA3-FUT6AS were constructed by *Hind*III digestion of pcDNA1-Fuc-TVI (H. Cameron et al., J. Biol. Chem., *270:* 20112-20122, 1995) and cloning of the insert (with opposite orientations) into pcDNA3. Finally, a truncated coding region antisense construct was created by amplification of FUT3 bp 733-1004 as described in Example 1 above. The resulting

10 272 bp fragment, which corresponds to the putative catalytic domain of FUT3 (j. Kukowska-Latallo et al., *Genes Devel.* **4**, 1288 (1990)) and FUT6 (B. Weston et al., *J. Biol. Chem.* **267**, 4152 (1992)), was cloned in reverse orientation in the *Hind*III site of pcDNA3 to yield the vector pcDNA3-FUT3CD AS, as described in Example 1 above.

Stable transfection, selection, and cloning of FUT3/FUT6 antisense and

15 **sense cell lines.** Approximately 24 hours before transfection, COLO-205 cells were plated at 50% confluence in flasks containing RPMI-1640 with 10% FBS. Cells were harvested and washed twice with PBS. In separate 4 mm gap cuvettes (Bio-Rad), 30 μg of the *Pvu*I-linearized plasmids pcDNA3-CAT, pcDNA3-FUT3S, pcDNA3-FUT3AS, pcDNA3-FUT6S, pcDNA3-FUT6AS, or pcDNA3-FUT3CD AS (above

20 and **Fig. 4**) were mixed with 10^7 cells in 500 μl of serum-free media and incubated for 10 minutes on ice. The cuvettes were then subjected to electroporation with $V_o =$ 380 volts and capacitance = 1000 μF on a Gene Pulser II apparatus (Bio-Rad). Following electroporation, the cells were immediately plated in 10 ml of RPMI-1640 with 10% FBS in 100 mm plates and incubated at 37^0 C in 5% CO_2.

25   Forty-eight hours after transfection, G-418 sulfate (GIBCO-BRL) was added to the medium at a concentration of 1 mg/ml. At 3-4 weeks in G-418, distinct colonies were observed on each plate and selected with 3 mm cloning cylinders (Fisher, Pittsburgh, PA). The colonies were cloned by limiting dilution in 96 well plates with continued G-418 selection. To assure a homogeneous population, the

30 resulting clones were again subjected to limiting dilution under G-418 selection. Four clonal sense, four clonal antisense, and three clonal CAT control cell lines were

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established. Representative clones of each were further characterized for glycan, enzyme, and transcript expression. The sense clone chosen was designated as S6G (transfected with pcDNA3-FUT3S and pcDNA3-FUT6S). The two antisense clones selected were AS5C (transfected with pcDNA3-FUT3AS and pcDNA3-FUT6AS) and

5   AS7A (transfected with pcDNA3-FUT3CD AS).

Proliferation and apoptosis assays. The proliferative ability of transfectants and controls was first measured in RPMI-1640 supplemented with 10% FBS. Transfectants were maintained in G-418 as described above. Cells were seeded at a concentration of 10^4 cells/cm^2. Medium renewal was performed every two days. A

10   flask from each combination was harvested daily, and cell yield was determined. Due to its slower growth rate, additional flasks of clone AS7A were harvested at each time point. For anchorage-independent proliferation (AIP) assays, cells were plated at 50,000 cells per 60 mm dish in 0.33% Noble agar (DIFCO) over a 7 ml base layer of 0.5% agar. The medium was supplemented with 5 μM putrescine and 0.1 μg/ml

15   DEAE-dextran (Sigma). One milliliter of medium was added to the dishes every week. Colony-forming efficiency (CFE) was determined after four weeks. For apoptosis assays, transfectants and controls were harvested for flow cytometry as described (B. Weston et al., J. Biol. Chem., *267*: 4152-4160, 1992). Annexin V and propidium iodide staining were performed according to the manufacturer's protocol

20   (R&D Systems) and unfixed cells were immediately analyzed. Experiments were performed twice for each cell line, n = 3 for each experiment (with the exception of clone AS7A, n = 2 for each experiment).

Tumorigenicity assays. NCR nu/nu athymic nude mice were obtained from Taconic (Germantown, N.Y.). Eight-week-old female nude mice weighing 20-22 gm

25   were injected subcutaneously with 10^7 cells in 100 μl PBS per flank site. Animals were monitored for tumor size over six to eight weeks or until the mass reached 1.5 cm in diameter. The animals were then sacrificed and the tumors harvested, examined, and weighed.

30   **B. RESULTS**

COLO-205 cells express high levels of sialyl Lewis x, sialyl Lewis a, α(1,3)fucosyltransferase activity, and FUT3/FUT6 transcripts. Results of flow

cytometry analyses of surface antigens on COLO-205 cells are summarized in **Fig. 8.** COLO-205 cells do not express high levels of the non-sialylated antigen, Lewis x, or the internally fucosylated antigen, VIM-2 (data not shown). High surface levels of the fucosylated and sialylated selectin ligands sialyl Lewis a and sialyl Lewis x are

5   noted. To assess α(1,3/1,4) fucosyltransferase activity, enzyme assays with low molecular weight carbohydrate acceptors were performed as described above. As shown in **Fig. 9**, cellular extracts from COLO-205 transferred fucose efficiently to both type I and type II acceptors. Northern blot analyses of COLO-205 cells were carried out (data not shown). Probes specific for FUT3 and FUT6 (H. Cameron et al.,

10   J. Biol. Chem., *270:* 20112-20122, 1995) show high levels of transcript in the parental cell line. Using similar conditions, expression of other human fucosyltransferase transcripts is low (FUT4) or not detectable (FUT5 and FUT7) (data not shown). This antigenic, enzymatic, and transcript expression profile is consistent with high levels of Fuc-TIII and Fuc-TVI in COLO-205. FUT3 and FUT6 were therefore chosen as the

15   primary targets for antisense inhibition experiments.

Characterization of FUT expression in COLO-205 antisense, sense, and control cell lines. Constructs used for stable transfection of COLO-205 cells are shown in **Fig. 3**. The plasmid pcDNA3-CAT was used throughout the following experiments to monitor expression levels over time by CAT assay (data not shown).

20   Enzyme assay results in **Fig. 9** show that transfection of the control plasmid pcDNA3-CAT did not affect fucosyltransferase activities. Stable expression of pcDNA3-FUT3S and pcDNA3-FUT6S sense constructs in clone S6G enhanced fucosyltransferase activity. In contrast, the cloned antisense cell lines AS5C and AS7A had less than 2% specific activity with sialyl LacNac, the trisaccharide

25   precursor for sialyl Lewis x, when compared to untransfected COLO-205 cells. Furthermore, extracts from antisense clones showed marked reduction in the ability to fucosylate the Type I acceptor Lacto-*N*-biose I. Flow cytometry analyses of surface antigens on COLO-205 and stable antisense transfectants (**Fig. 8**) confirmed that the synthesis of sialyl Lewis x and sialyl Lewis a is inhibited in the antisense clones.

30   Northern blot analyses show that FUT3 and FUT6 transcript levels are markedly reduced in both stable antisense transfectant lines (data not shown).

Proliferation of antisense-transfected cells is impaired. The proliferative

ability of control, sense, and antisense transfectants was compared in media

containing 10% FBS. The growth rate of parental (untransfected) and control

(pcDNA3-CAT-transfected) COLO-205 cells was similar (**Fig. 10**). Sense clone S6G

5   grew at a notably higher rate, while antisense cell line AS5C grew more slowly than

control. Antisense AS7A proliferated at such a slow rate that additional cells were

maintained just to allow expression studies to be performed (**Fig. 10**). Representative

photomicrographs of G-418-selected colonies at day 28 were taken (not shown); no

definite change in morphology of the transfectants is noted. Anchorage independent

10   proliferation (AIP) was inhibited by expression of antisense FUT constructs (**Table**

4). Conversely, AIP of sense transfectants was higher than control transfectants and

parental COLO-205. Flow cytometric analyses of control, sense, and antisense lines

showed no differences in the proportions of apoptotic (annexin-V-positive) or non-

viable (propidium-iodide-positive) cells (data not shown).

15   **COLO-205 cells transfected with FUT3/FUT6 antisense sequences are less**

tumorigenic in nude mice. The results of *in vivo* growth assays employing flank

injections of nude mice with control and transfectant cell lines are summarized in

Table 5. Parental (untransfected) COLO-205 are highly tumorigenic (T. Kuo et al.,

Proc. Natl. Acad. Sci. USA, *92:* 12085-12089, 1995). Sense transfectants showed

20   slightly higher growth rates in this assay, although the number of mice injected was

small for comparisons. Antisense transfectant lines AS5C and AS7A grew at

markedly slower rates during the course of the study. These data indicate that

expression of antisense FUT sequences is capable of inhibiting colon carcinoma

growth *in vivo*.

25

Table 4. Anchorage-independent proliferation of COLO-205 and transfectant cell lines.

Cell line	CFE (%)
COLO-205	10.7 +/- 1.2
pcDNA3-CAT	12.0 +/- 1.5
S6G	26.8 +/- 3.3
AS5C	3.46 +/- 0.7
AS7A	2.72 +/- 0.7*

*n = 2 for both experiments (n = 3 for other lines).
Colony-forming efficiency (CFE) is reported as the mean +/- SD of the pooled observations from both experiments.

Table 5. Tumorigenicity of COLO-205 and transfectant cell lines.

Cells injected	#Mice	Tumor wts. (mg)
COLO-205	3	470 (433-504)
S6G	4	682 (605-740)
AS5C	3	48 (30-78)
AS7A	3	35 (0-80)

Cells were injected subcutaneously in nude mice as described in Materials and Methods. Tumors were harvested and weighed at six to eight weeks or when 1.5 cm in diameter. Weights are reported as average milligrams (mg) and range.

The foregoing is illustrative of the present invention, and is not to be construed as limiting thereof. The invention is defined by the following claims, with equivalents of the claims to be included therein.